Vol. 109, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1982 Pages 186-193

IDENTIFICATION OF THE COATED VESICLE PROTEINS THAT BIND CALMODULIN

Carol D. Linden

Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

Received October 6, 1982

SUMMARY: The constituent proteins of coated vesicles responsible for binding calmodulin were identified by photoaffinity labeling with the reagent azido-^{125}I-calmodulin. Three protein complexes with apparent molecular weights of 130,000, 93,000 and 52,000 were labeled. Specificity was demonstrated by the dependence of labeling on Ca^{2+}, and by its reduction in the presence of unlabeled calmodulin or Stelazine. Urea-soluble components of coated vesicles and material isolated by Sepharose CL4B chromatography formed a 52,000 MW labeled complex. Subtracting an apparent molecular weight of calmodulin of 20,000 from the weights of the covalently labeled complexes, the coated vesicle proteins that bind calmodulin are 110,000, 73,000 and 32,000 MW. The 32,000 MW protein is thought to participate in the coat structure but the other two are most likely associated with the vesicle.

Calmodulin (CAM), a ubiquitous calcium-binding regulatory protein, has been characterized widely with respect to its ability to modulate various enzymatic activities and cellular structural assemblies (for review see refs. 1-5). In addition, it was demonstrated recently (6) that CAM binds to purified coated vesicles in vitro, although a biological function attributable to this interaction has not been determined. The studies of Salisbury et al. (7) suggest that CAM is involved in the formation of coated pits. These authors showed that Stelazine, a known inhibitor of CAM function, inhibits the formation of coated pits in apposition to clustered IgM on the surfaces of WiL2 cells. Although the protein composition of coated vesicles (and, by inference, coated pits) has been well characterized (8-13), previous efforts to determine which of the coated vesicle proteins recognize CAM showed only that clathrin, the principal structural coated vesicle protein, did not bind CAM appreciably (6).

Here, the CAM binding proteins of coated vesicles were identified using the photoaffinity probe, azido-^{125}I-CAM, developed by Andreasen et al. (16). This reagent

The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

0006-291X/82/210186-08$1.00/0

allows direct covalent labeling of CAM binding proteins and was used here to label constitutent polypeptides of purified coated vesicles. The results of these experiments show that azido-^{125}I-CAM and coated vesicle proteins form covalent complexes with approximate molecular weights of 130,000, 93,000, and 52,000.

MATERIALS AND METHODS:

Purifications: Coated vesicles were purified as described previously (6) from locally obtained bovine brain which had been stored at -70°C. The isolation buffer contained 0.1M MES, 1mM EGTA, 0.5 mM $MgCl_2$, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 6.5. The protein content of purified coated vesicles was determined using the Bio Rad protein determination kit with bovine serum albumin as a standard.

Calmodulin was purified from bovine brain using the procedure described by Schreiber et al. (17). Purity and biological activity were assessed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (18) and stimulation of erythrocyte Ca^{2+} + Mg^{2+}-ATPase (19) respectively. Calmodulin was quantitated spectrophotometrically using a value of $E^{1\%}_{280}$ = 21 (20).

Fractionation of Solubilized Coated Vesicles: Coated vesicles were solubilized using isolation buffer containing 2M urea (8) and fractionated by chromatography on Sepharose CL4B (11). Coated vesicles (7 mg) were pelleted, resuspended in 2M urea buffer and incubated for 1 hr at room temperature. Soluble material was separated from insoluble material by centrifugation. The supernatant was chromatographed on a Sepharose CL4B column (1.2 x 18cm) in 2M urea buffer and the pellet was resuspended in isolation buffer containing 0.1% Triton X-100. Aliquots of the urea-soluble material and the peak fraction obtained from the chromatography were dialyzed 18 hr at 4°C against isolation buffer to promote reassembly of coats.

Photoaffinity Labeling: Azido-^{125}I-CAM, produced by derivitizing ^{125}I-CAM with methyl-4-azidobenzoimidate (16), was the generous gift of Dr. Terry Andreasen. Coated vesicles (40 µg) or samples of solubilized and chromatographed material were incubated with this reagent in a total volume of 50 µl containing 0.1 M MES, pH 6.8, 1 mM EGTA and, as required, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 200 µM Stelazine (Smith, Kline and French Labs, Philadelphia, Pa.) or 25 µM unlabeled CAM. Reaction mixtures were allowed to incubate at room temperature for 1 hr in the dark. Samples were photolyzed on ice for 3-5 min using a Mineral-light UVS-11 held 1 cm from the sample. After photolysis, the samples were pelleted at 130,000 g for 15 min in a Beckman Airfuge, the supernatant containing unreacted azido-^{125}I-CAM removed, and the pellet solubilized in 50 µl of 0.1% SDS. Solubilized pellets or supernatants were mixed with sample buffer, heated and applied immediately to a 10% SDS-polyacrylamide slab gel (18). The gel was stained with Coomassie blue and destained as described (8), then dried onto filter paper and exposed at -70°C to Kodak X-OMAT AR X-ray film backed by an intensifying screen.

RESULTS:

Direct covalent labeling of intact coated vesicles with azido-^{125}I-CAM allowed a more definitive identification of the coated vesicle proteins which recognize CAM. In contrast to the affinity chromatography experiments presented previously (6), this technique did not require the dissociation of the coated vesicles with urea nor the presence of urea during the experiment. Thus, the interaction of CAM with coated vesicles could be examined in the absence of a denaturing agent. Figure 1 shows the results obtained when azido-^{125}I-CAM was incubated with coated vesicles under

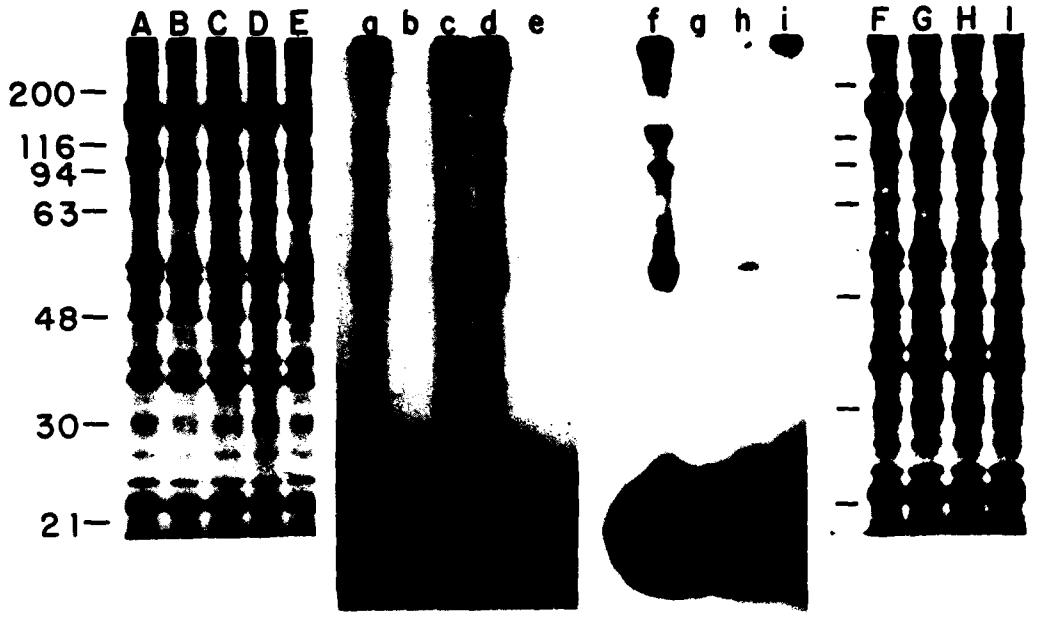

Figure 1: SDS-polyacrylamide gel (A-I) and corresponding autoradiograph (a-i) of coated vesicles exposed to azido-^{125}I-CAM. Standard conditions are described in Methods; additions are indicated here. Samples A-E (a-e) contained 250 nM reagent; samples F-I (f-i) contained 125 nM reagent A (a) 2 mM $CaCl_2$; B (b) 2 mM $MgCl_2$; C (c) 2 mM $MnCl_2$; D (d) same as A; E (e) same as A except that sample was not photolyzed; F(f) same as A except using the lower concentration of reagent; G (g) $CaCl_2$ omitted; H (h) 2 mM $CaCl_2$ and 25 μM native CAM; I (i) 2 mM $CaCl_2$ and 200 μM Stelazine. The overexposed area at the bottom of the autoradiographs corresponds to azido-^{125}I-CAM.

conditions designed to promote binding and under conditions were binding was expected to be inhibited. In the presence of free Ca^{2+}, azido-^{125}I-CAM covalently labeled coated vesicle polypeptides to form complexes with approximate molecular weights of 130,000, 93,000, and 52,000 (lanes a, d, f). In addition, material at the top of the gel with a molecular weight in excess of 300,000 was labeled. In the absence of Ca^{2+}, no labeling was observed (lane g). The overexposed area at the bottom of the autoradiographs corresponds to azido-^{125}I-CAM. Substitution of manganese for calcium resulted in a labeling pattern identical to, although not as intense as, that observed in the presence of calcium (lane c) whereas substitution of magnesium as the divalent cation did not result in labeling (lane b). If excess native CAM (25 μ M) was added to the coated vesicles 30 min prior to the addition of azido-^{125}I-CAM, labeling was greatly reduced (lane h). Inclusion of Stelazine (200 μ M), a CAM inhibitor (1, 3, 21-23), also reduced labeling to a low level (lane i) relative to that observed in the absence of the drug.

Figure 2: SDS-polyacrylamide gel (A-I) and corresponding autoradiograph (a-i) of solubilized and fractionated coated vesicles reacted with 25 nM azido-^{125}I-CAM in the presence of 2mM $CaCl_2$. Experimental details are described in methods. A (a) intact coated vesicles; B (b) intact coated vesicles, calcium omitted from assay; C (c) coats reassembled from urea-soluble material by dialysis against isolation buffer and pelleted in the labeling assay; D (d) peak material from Sepharose CL4B chromatography dialyzed and pelleted in labeling assay; E (e) urea-insoluble pellet from initial extraction of coated vesicles; F (f) intact coated vesicles plus 25 μM unlabeled CAM; G (g) supernatant of sample C from labeling assay; H (h) supernatant of sample D from labeling assay; I (i) supernatant of sample E from labeling assay.

In an effort to differentiate between CAM recognition proteins associated with the coat versus those associated with the vesicle, purified coated vesicles were solubilized with urea and chromatographed on Sepharose CL4B. Samples of the resulting fractionated material were then exposed to azido-^{125}I-CAM. The results obtained from solubilization and chromatography in 2M urea-isolation buffer are shown in Fig. 2. Labeling of intact coated vesicles was identical to that shown in Fig. 1 (lanes A, a), with no labeling observed in the absence of calcium (lanes B, b) or in the presence of excess unlabeled CAM (lanes F,f). Prior to chromatography, the urea solubilized coated vesicles were centrifuged to separate soluble from insoluble (pellet) material. The pellet obtained at this step had a protein profile and labeling profile virtually identical to that of intact coated vesicles (lanes E,e). When this material was re-centrifuged as part of the photoaffinity labeling procedure, however, the soluble material remaining in the

supernatant contained clathrin, the two 30,000 dalton proteins and proteins of ca 100,000 and 50,000 MW (lane I). This material, however, did not become covalently labeled with the azido-^{125}I-CAM. An aliquot of the original urea soluble material reassembled to form coats after dialysis against isolation buffer, as evidenced by the protein profile of this material obtained from the pellet in the labeling assay (lane C). Interestingly, the reassembled coats failed to label (lane c) but the material remaining in the supernatant (lanes G,g) showed a distinctive labeling pattern. In contrast to intact coated vesicles, a complex of ca 52,000 MW was labeled prominently with additional labeling of a ca 100,000 MW protein and low MW material (> 30,000) also observed. Similarly, material obtained from chromatography on Sepharose CL4B failed to reassemble to form pelletable coats after dialysis against isolation buffer (lanes D,d). After processing in the labeling assay, this material contained only clathrin plus trace amounts of the 30,000 MW proteins (lane H). Virtually the only labeling observed (lane h) was a ca 52,000 MW complex with 3 faint but distinct higher MW species also apparent. Separate experiments confirmed that the labeling of this material was Ca^{2+} dependent and inhibited by excess unlabeled CAM (not shown). Similar labeling results were obtained using material that was solubilized and fractionated according to the procedures described by Ungewickell and Branton (15).

DISCUSSION:

Direct identification of the coated vesicle proteins that recognize CAM was accomplished using a photoactivatible derivative of ^{125}I-CAM, azido-^{125}I-CAM (16). The biological activity and ability of this reagent to form covalent linkages with several CAM-regulated proteins has been demonstrated (16). It was used here to label specifically three protein constituents of coated vesicles. The specificity of labeling was demonstrated by its dependence on Ca^{2+} or Mn^{2+} but not Mg^{2+}, and its inhibition by excess unlabeled CAM and by Stelazine. The residual labeling seen in the presence of CAM and Stelazine may represent the nonspecific binding of CAM to coated vesicles which has been described (6). Although the issue of nonspecific binding is not readily resolved using this experimental system, it is clear that the interaction of azido-^{125}I-CAM with coated vesicles is strictly calcium-dependent and fulfills the

classical criteria for assessing specific CAM interactions with various proteins (1-5). A very high molecular weight component which barely enters the 10% polyacrylamide gel also labeled specifically. The significance of this component is unclear; it was not removed by low speed centrifugation of the coated vesicles prior to exposure to the azido-^{125}I-CAM, and probably represents a high molecular weight contaminant known to copurify with coated vesicles (8).

The results of photoaffinity labeling of solubilized and fractionated coated vesicles suggest that discrete components of both the coat and the vesicle recognize CAM. It also appears that the conformation of these components may be critical to binding CAM. As shown in Fig. 2, coated vesicles and urea-insoluble material derived from them labeled identically with azido-^{125}I-CAM. Coats reassembled from urea-soluble components, however, did not bind the photoaffinity label (lanes C,c) but soluble material (lane G) that failed to form pelletable coats formed a major covalently labeled complex of MW 52,000 and one with MW 116,000. Heterogeneous material > 30,000 MW was also labeled (lane g). This labeling pattern is quite different from that of intact coated vesicles despite the similarities in the protein composition of the two samples. The labeling of urea-soluble material which had been chromatographed on Sepharose CL4B was even more striking. In this case, material from the column contained only clathrin plus trace amounts of the two 30,000 MW "light chains", and one principal species of 52,000 MW was labeled with azido-^{125}I-CAM. Again, this material had failed to form pelletable coats after dialysis against isolation buffer. In contrast to urea-soluble "coat material", the urea-insoluble "pellet", which contains vesicles plus other adherent proteins, displayed different labeling properties. As shown in fig. 2, (lanes I,i), a certain amount of pellet material remained in the supernant after recentrifugation during the labeling assay. This supernatant contained clathrin, the two 30,000 MW light chains, proteins of ca 100,000 and 2 proteins ca 50,000 MW, and yet no covalent labeling to this material was observed. Assuming that the prominently labeled 52,000 MW complex seen in urea-soluble material represents the crosslinked product of CAM and one of the 30,000 MW light chains, it seems that this protein does not label under all conditions but probably only in a particular conformation. It is not clear why labeling occurred in intact coated vesicles but not in reassembled coats.

The azido-^{125}I-CAM-protein complexes formed in intact coated vesicles migrated on a 10% SDS gel with apparent molecular weights of 130,000, 93,000, and 52,000. In this gel system, CAM itself migrates with an apparent molecular weight of 20,000. Assuming that 1) the molecular weights of CAM and the crosslinked proteins are additive in terms of migration on an SDS-gel (16) and that 2) there is a one-to-one stoichiometry between azido-^{125}I-CAM and its binding proteins, the molecular weights of the coated vesicle proteins that recognize CAM are 110,000, 73,000, and 32,000. The assumption of a one-to-one stoichiometry is supported by the observation that the same labeling pattern of coated vesicles is obtained using azido-^{125}I-CAM at 25 nM (Fig. 2), 125 nM or 250 nM (Fig. 1). Because the efficiency of crosslinking between azido-^{125}I-CAM and known CAM binding proteins ranges between $\simeq$ 25 and 40% depending on the protein (16), it is difficult to determine the stoichiometry of binding with precision.

The results presented here show that a limited number of coated vesicle proteins recognize CAM in a specific manner. With the exception of the 32,000 MW protein, these proteins are not intrinsic to the coat structure (8, 9, 11, 13-15, 24) but may play important roles in the coat interaction with membrane or in the interaction of a coated pit or vesicle with cytoskeletal elements. In particular, a 110,000 MW protein associated with the vesicle appears to mediate the formation of coats around the vesicle (25). The 32,000 MW protein is a component of triskelion coat subunits and appears to be required for formation of the coat structure (11, 15). The roles of CAM association with coated vesicle proteins and of the proteins themselves remain to be determined.

ACKNOWLEDGMENTS:

The author thanks Mr. Robert Bolton for excellent technical assistance and Dr. Terry Andreasen for the generous gift of azido-^{125}I-CAM and many fruitful discussions.

REFERENCES:

1. Cheung, W.Y. (1980) Science 207, 19-27.
2. Wang, J.M., and Waisman, D.M. (1979) Curr. Top. Cell. Regul. 15, 47-107.
3. Wolff, D.J., and Brostrom, C.O. (1979) Adv. Cyclic Nucleotide Res. 11, 27-88.
4. Klee, C.B., Crouch, T.H., and Richman, P.G. (1980) Annu. Rev. Biochem. 49, 489-515.
5. Means, A.R., and Dedman, J.R. (1980) Nature 285, 73-77.
6. Linden, C.D., Dedman, J.R., Chafouleas, J.G., Means, A.R., and Roth T.F. (1981) Proc Natl. Acad. Sci. USA 78, 308-312.
7. Salisbury, J.L., Condeelis, J.S., and Satir, P. (1980) J. Cell Biol. 87, 132-141.
8. Woodward, M.P., and Roth, T.F. (1978) Proc. Natl. Acad. Sci. USA 75, 4394-4398.

9. Pearse, B.M.F. (1976) Proc. Natl. Acad. Sci. USA 73, 1255-1259.
10. Woods, J.W., Woodward, M.P., and Roth, T.F. (1978) J. Cell Sci. 30, 87-97.
11. Keen, J.H., Willingham, M.C., and Pastan, I.H. (1979) Cell 16, 303-312.
12. Rubenstein, J.L.R., Fine, R.E., Luskey, B.D., and Rothman, J.E. (1981) J. Cell Biol. 89, 357-361.
13. Pearse, B.M.F. (1978) J. Mol. Biol. 126, 803-812.
14. Schook, W., Puszkin, S., Bloom, W., Ores, C., and Kochwa, S. (1979) Proc. Natl. Acad. Sci. USA 76, 116-120.
15. Ungewickell, E., and Branton, D. (1981) Nature 289, 420-422.
16. Andreasen, T.J., Keller, C.M., LaPorte, D.C., Edelman, A.M., and Storm, D.R. (1981) Proc. Natl. Acad. Sci. USA 78, 2782-2785.
17. Schreiber, W.E., Sasagawa, T., Titani, K., Wade, R.D., Malencik, D., and Fischer, E.M. (1981) Biochemistry 20, 5239-5245.
18. Laemmli, U.K. (1970) Nature 227, 680-685.
19. Jarrett, H.W., and Kyte, J. (1979) J. Biol. Chem. 254, 8237-8244.
20. Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D., and Means, A.R. (1977) J. Biol. Chem. 252, 8415-8422.
21. Charbonneau, H., and Cormier, M.J. (1979) Biochem. Biophys. Res. Commun. 90, 1039-1047.
22. Jamieson, G.A., Jr., and Vanaman, T.C. (1979) Biochem. Biophys. Res. Commun. 90, 1048-1056.
23. Roufogalis, B.D. (1981) Biochem. Biophys. Res. Commun. 98, 607-613.
24. Kartenbeck, J. (1978) Cell Biol. Int. Rep. 2, 457-464.
25. Unanue, E.R., Ungewickell, E., and Branton, D. (1981) Cell 26, 439-446.